FEMS Microbiology Reviews, fuv005, 39, 2015, 541–554

doi: 10.1093/femsre/fuv005
Advance Access Publication Date: 15 April 2015
Review Article

REVIEW ARTICLE

Small things considered: the small accessory subunits of RNA polymerase in Gram-positive bacteria

Andy Weiss and Lindsey N. Shaw*

Department of Cell Biology, Microbiology and Molecular Biology, University of South Florida, Tampa, FL 33620, USA

*Corresponding author: Department of Cell Biology, Microbiology and Molecular Biology, University of South Florida, 4202 East Fowler Avenue, ISA 2015, Tampa, FL 33620-5150, USA. Tel: 813-974-2087; Fax: 813-974-1614; E-mail: shaw@usf.edu

One sentence summary: This review details over four decades of research on the small, accessory subunits of the Gram-positive transcriptional machinery.

Editor: Grzegorz Wegrzyn

ABSTRACT

The DNA-dependent RNA polymerase core enzyme in Gram-positive bacteria consists of seven subunits. Whilst four of them ($\alpha_2\beta\beta'$) are essential, three smaller subunits, δ, ε and ω (~9–21.5 kDa), are considered accessory. Both δ and ω have been viewed as integral components of RNAP for several decades; however, ε has only recently been described. Functionally these three small subunits carry out a variety of tasks, imparting important, supportive effects on the transcriptional process of Gram-positive bacteria. While ω is thought to have a wide range of roles, reaching from maintaining structural integrity of RNAP to σ factor recruitment, the only suggested function for ε thus far is in protecting cells from phage infection. The third subunit, δ, has been shown to have distinct influences in maintaining transcriptional specificity, and thus has a key role in cellular fitness. Collectively, all three accessory subunits, although dispensable under laboratory conditions, are often thought to be crucial for proper RNAP function. Herein we provide an overview of the available literature on each subunit, summarizing landmark findings that have deepened our understanding of these proteins and their function, and outline future challenges in understanding the role of these small subunits in the transcriptional process.

Keywords: RNA polymerase; delta subunit; RpoE; omega subunit; RpoZ; epsilon subunit; RpoY; transcriptional regulation

INTRODUCTION

The ability of bacterial cells to precisely adjust and adapt to their environment is crucial for survival. Accordingly, the expression of genes, and their products that facilitate adaptation to changing conditions, is a highly controlled and organized process. Transcription in all forms of life is performed by DNA-dependent RNA polymerase (RNAP), with enzymes from the different branches of life showing a high degree of similarity (Ebright 2000; Sekine, Tagami and Yokoyama 2012). With respect to eubacteria, core RNAP consists of two α subunits, one β and one β' subunit, all of which are essential for a viable cell. Besides these well-studied and essential components, several additional, smaller subunits, δ, ε and ω, have been described and intensively researched over a number of decades (Burgess 1969; Pero, Nelson and Fox 1975; Keller *et al.*, 2014). Additional to this, bacteria possess σ factors (housekeeping or alternative) that assist RNAP with promoter recognition, and the initiation of transcription (Helmann and Chamberlin 1988; Feklistov *et al.*, 2014). While $\alpha_2\beta\beta'$ RNAP and the σ factor together are able to perform all of the required steps for transcription, the additional, smaller subunits support the complex by various means, reaching from coordinating RNAP folding and assembly to increased transcriptional specificity, influencing RNAP recycling and possibly protecting the cell against phage infection.

Received: 19 December 2014; **Accepted:** 19 January 2015

As ω is found in Gram-positive as well as Gram-negative bacteria (Minakhin *et al.*, 2001), it has been more heavily investigated than the other subunits (for a thorough review, see Mathew and Chatterji 2006). With regard to the δ subunit, it is seemingly confined to the Firmicutes; although proteins with some sequence homology can be found in certain Tenericutes as well (our unpublished observation). Collectively, despite many years of research on this protein in low G + C Gram-positive bacteria, the literature documenting it has never been collated in a single place. As such, it forms a major component of the work detailed herein. Nevertheless, we also seek to also update the literature surrounding ω, as well as introduce newly published data on ε. We believe that of these subunits, δ is particularly interesting, since we still lack a complete understanding of how this enigmatic protein mediates its function, despite a history of more than 40 years of research. Accordingly, we present the progress of δ research over four decades, summarizing key milestones in our understanding of this subunit, and highlight future challenges in dissecting its role in transcription, as well as cellular fitness and survival.

THE δ SUBUNIT OF RNAP

Structure, abundance and interaction of the δ subunit with RNAP

The interaction of δ with RNAP

The presence of δ as an integral part of RNAP was first described in 1975 when Pero, Nelson and Fox (1975) identified and named a 21.5 kDa protein that co-purified with the RNAP complex from phage-infected *Bacillus subtilis*. Here the group reported for the first time the effects of δ on transcriptional specificity, using *in vitro* transcription assays that demonstrated it was required to maintain the strand-specific transcription of phage genes characteristically observed during *in vivo* infection. These experiments provided not only a structural but also a functional link between RNAP and δ. Subsequently, several groups simultaneously described the co-purification of δ with RNAP in *B. subtilis* (Halling, Burtis and Doi 1977; Plevan *et al.*, 1977; Tjian *et al.*, 1977). In a more recent approach, Doherty *et al.* (2010) used fluorescent-labeled δ in *B. subtilis* to observe its interaction with RNAP *in vivo*, showing the subcellular co-localization of this subunit with other RNAP proteins (β' and ω). Lately, our group demonstrated the interaction of δ with the β and β' subunits of RNAP under native conditions in *Staphylococcus aureus* (Weiss *et al.*, 2014). This interaction is most likely facilitated by the N-terminus of the protein, since a C-terminally truncated version of the enzyme still co-purifies with core RNAP (Lopez de Saro, Woody and Helmann 1995). Stoichiometrically, it appears that the subunit binds in an approximate 1:1 ratio with other subunits of core-RNAP (the α subunit excepted) (Halling, Burtis and Doi 1977). Interestingly, early studies were unable to purify RNAP containing a σ factor as well as δ at the same time. This led to the idea of temporally separated binding of these two subunits, and questions about when and how δ interacts with core RNAP. In order to understand the association of δ with core RNAP in a time-dependent manner, and in correlation to DNA-binding and transcriptional initiation, sedimentation experiments were used, demonstrating that excess amounts of the subunit lead to release of the σ factor from *B. subtilis* RNAP (Williamson and Doi 1978). This seemed plausible since δ had been shown in early studies to decrease activity of core RNAP at certain promoters, while σ is well known to have stimulatory effects, suggesting a competitive relationship between these two subunits. These findings, to a certain extent, were overruled when experiments showed the need for both subunits in the context of promoter selection (Achberger and Whiteley 1981), leading to key studies revealing that δ and σ factors are in fact able to bind to RNAP at the same time (Hyde, Hilton and Whiteley 1986). This ultimately excluded the hypothesis of a completely competitive δ vs σ relationship. Instead, the decreased binding ability of σ after δ subunit association with RNAP indicates 'negative cooperativity' in which both proteins are able to bind to the RNAP simultaneously, but where binding of δ possibly weakens the binding of σ and leads to increased release of the factor. Such a scenario suggests a model in which both subunits are required for directed binding to promoter regions, and initiation of transcription.

δ expression and protein levels within the cell

Various studies have explored the conditions that affect δ abundance within bacterial cells, using a variety of different strains and backgrounds. In general, there is agreement that *rpoE* (the δ encoding gene) is highly expressed during exponential phase (mid and late) and, to varying degrees, during stationary phase, in standard laboratory conditions. In *S. aureus*, peak expression appears to be during exponential phase under standard conditions and when grown in amino acid- or glucose-limiting media (Watson, Antonio and Foster 1998; Weiss *et al.*, 2014). In *B. subtilis*, *rpoE* is most highly expressed during the transition between exponential and stationary phase, as well as in extracts from spores (Lopez de Saro, Yoshikawa and Helmann 1999). For *Streptococcus agalactiae*, findings from reporter constructs mirror that seen in *S. aureus* and *B. subtilis*, with *rpoE* being expressed during all growth phases, peaking during lag and (late) log, before dropping slightly when reaching stationary phase (Seepersaud *et al.*, 2006). In *S. mutans*, it was shown that *rpoE* is expressed maximally during late exponential phase, and subsequently declines during stationary phase (Xue *et al.*, 2010). One study suggested that δ accounts for 0.3% ($\pm$ 0.1%) by weight of the total soluble protein of *B. subtilis* cells (10^4 molecules cell^{-1}), representing a 5:1 molar excess compared to RNAP during exponential and stationary growth phase (Lopez de Saro, Yoshikawa and Helmann 1999). In contradiction, Doherty *et al.* (2010) found a total abundance of 2.1×10^4 ($\pm$ 580) molecules per cell for the β' subunit and 2.3×10^4 ($\pm$ 900) molecules for δ in exponentially growing cells, resulting in an approximate 1:1 ratio of the subunit to RNAP. Although these studies show variation in comparison to each other, it is clear that δ is present in relatively equal amounts to other components of core RNAP, therefore suggesting a permanent interaction of this subunit with the transcription machinery. In terms of factors that influence its expression, it has been reported for *B. subtilis* (Lopez de Saro, Yoshikawa and Helmann 1999), *S. agalactiae* (Seepersaud *et al.*, 2006) and *S. aureus* (Weiss *et al.*, 2014) that *rpoE* transcription is driven from a σ^A-dependent promoter. Beyond this, *rpoE* does not appear to be autoregulated in *B. subtilis*, but is at least partially repressed by acid and H_2O_2 stress (Lopez de Saro, Yoshikawa and Helmann 1999). Collectively, the strong presence of δ during most/all growth phases, in multiple bacterial species, highlights its potential importance for Gram-positive cells.

Structure, domains and active sites of the δ subunit of RNAP

When studying the primary amino acid (aa) sequence of δ proteins from numerous organisms (Fig. 1), it is apparent that they all possess two distinct regions, as first described by Lopez de Saro, Woody and Helmann (1995): an ordered and structured N-terminus, and a flexible and unstructured C-terminus. The C-terminus is characterized by highly acidic and repetitive aa

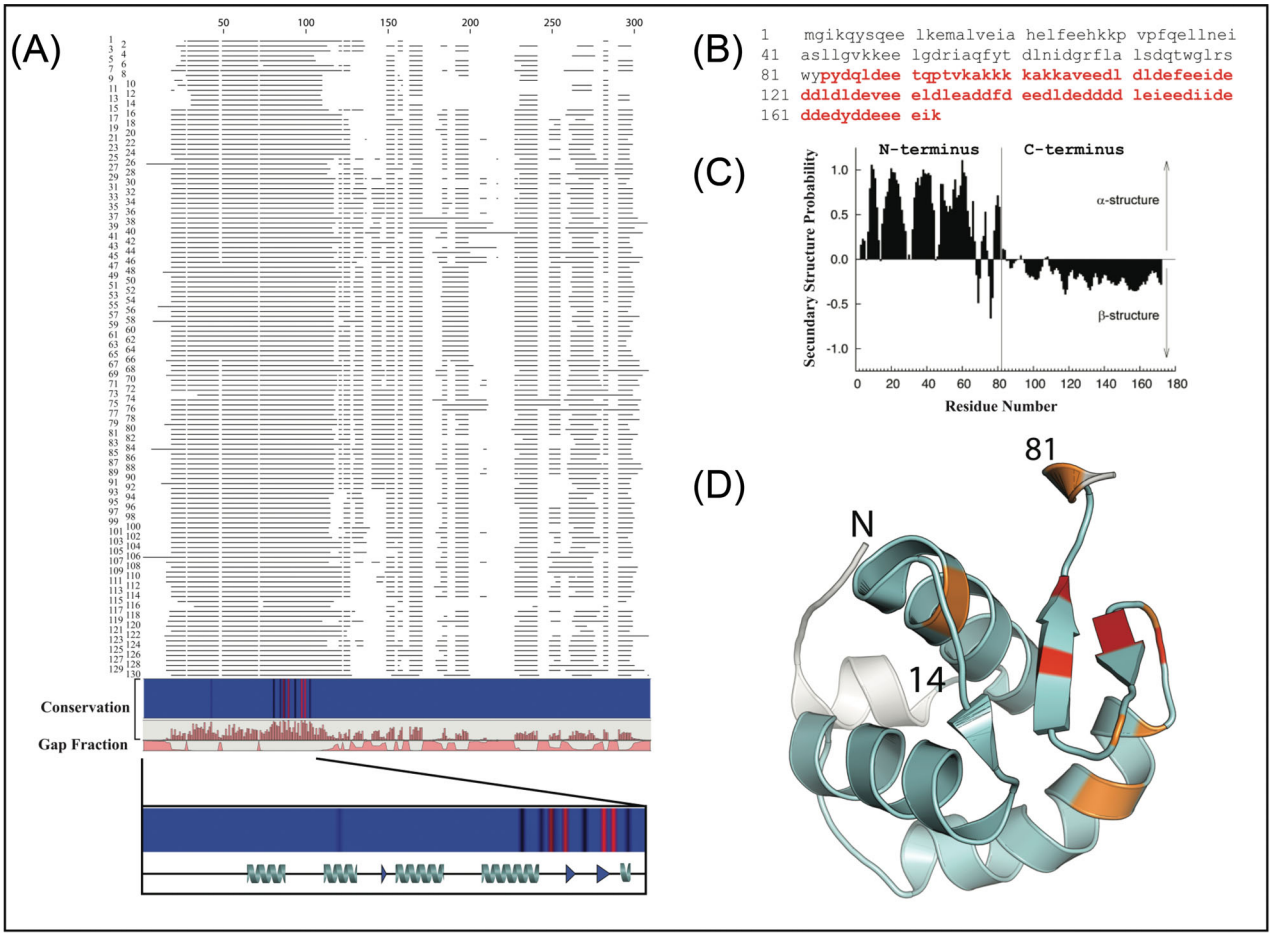

Figure 1. Structural features of the δsubunit of RNAP. **(A)** Alignments of 130 representative δ proteins from different Gram-positive species (using CLC Main Workbench software). Shown are (from top to bottom) broken black lines next to numbers from 1 to 130 (corresponding to bacterial species and IMG Gene ID numbers (Markowitz *et al.*, 2014), see Table S1, Supporting Information) represent the alignment of δ proteins. Sequence conservation is highlighted by two graphs: the upper one marks residues that are at least 95% (black) and 99% (red) conserved. The one below highlights the amount of conservation (0–100%) for each amino acid. The gapped fraction visualizes the amount of divergence in each region of the protein. At the bottom, an enlargement of the N-terminus is shown and highlights the conservation in regions that harbor specific protein domains (α-helices or β-sheets). **(B)** Amino acid sequence of the *B. subtilis* δ subunit. Black letters mark the N-terminus and red letters the C-terminus of the protein. **(C)** Secondary structure prediction of the ordered N-terminus and the disordered C-terminus of the δ protein, showing a bias towards α and β structures, respectively. This figure is reproduced from Papouskova *et al.* (2013). **(D)** Structure of *B. subtilis* δ from the protein database (PDB ID: 2M4K). The blue region corresponds to the HARE-HTH domain (residue 14–81); residues in red (≥99%) and orange (≥95%) highlight amino acids that are conserved from the alignment.

residues; together, the N- and C-termini have a significantly acidic pI of 3.6. A wealth of additional structural information for δ was generated by Motackova *et al.* (2010) using NMR technology and the *B. subtilis* protein, specifically focusing on the N-terminal 100 aa. Four α-helices were identified; each being 5 to 12 aa in length (residue Q8–K12, L16–H27, F33–L44, G52– N63). In addition, a β-sheet, consisting of three β-strands (residues V31–P32, F68–A70, T75–L78), was reported. A concern with these approaches was the inclusion of a His-tag to facilitate purification, possibly interfering with NMR analysis. To assess this, the same group later conducted structural analyses on the full-length protein lacking such a tag, again using NMR (Papouskova *et al.*, 2013). This approach proved to be especially challenging because of the acidic C-terminus; however, these studies confirmed previous work with the His-tagged N-terminal variant. Relaxation experiments in these works showed that the C-terminal tail transiently contacts the ordered N-terminal domain, leading the authors to suggest that folding of the N-terminus may be influenced by interactions with its C-terminal partner. In addition, interdomain interactions (between the C- and N-terminus, and the C-terminus with itself), as well as the formation of possible β-sheet structures, were predicted for the flexible C-terminal region.

An attempt to identify functional domains found only weak homology to DNA/RNA-binding proteins for the N-terminus of δ, and therefore initially no nucleic acid binding properties of the subunit were assumed (Motackova *et al.*, 2010). A more recent study then compared sequence and structural features of a variety of elements to the human ASXL protein, identifying similarities of the ASXL N-terminus and δ (Aravind and Iyer 2012). The N-terminus of this protein is characterized by a winged helix-turn-helix (wHTH) domain that was named after its presence in the group of **H**B1, **A**SXL and **r**estriction **e**ndonuclease proteins (HARE-HTH). Other than the δ subunit, HARE-HTH domains in prokaryotes are mainly found in restriction endonucleases that are associated with DNA-modifying methylases. Based on this study, a role in the recognition of modified DNA sequences for δ has been suggested, although it is not yet experimentally

proven. Nevertheless, the presence of a HARE-HTH motif in δ presents the interesting possibility that not only does it interact with RNAP but that it also contacts DNA itself, allowing it to discriminate between sequences that differ, for example, in their degrees of DNA modification. As such, it is tempting to speculate that, similar to alternative σ-factors, δ potentially mediates directed transcriptional effects by recognizing specific promoter features, rather than solely having global effects on a large number of genes through modulation of RNAP activity.

To understand the importance and conservation of key δ regions, our group recently published alignments using various different species that carry a δ subunit protein (Weiss *et al.*, 2014) (Fig. 1). We demonstrate that HARE-HTH domains show a higher degree of conservation than other portions of the protein, emphasizing its likely importance for function. In contrast, the C-terminus of δ shows a large degree of variation. Further to this, there is a clear degree of divergence in the context of protein length for δ from various species (Table S1, Supporting Information), largely resulting from variations in the C-terminal domain. This suggests that the C-terminus either plays species specific roles or is at least partially dispensable for full function of the subunit. In line with this latter idea, we have shown that a δ variant of *S. aureus* containing a truncated C-terminus (missing ~25% of the protein) is still able to successfully interact with components of the transcription machinery, and complement, at least in part, the phenotypes of an *rpoE*-null strain (Weiss *et al.*, 2014). This is in line with earlier findings in *B. subtilis*, also describing that interaction of δ with RNAP is mediated by the N-terminus of the subunit (Lopez de Saro, Woody and Helmann 1995).

The δ subunit and its impact on transcriptional selectivity

Early work highlighting the influence of δ on transcription

The majority of early information regarding δ function was generated by studies exploring transcriptional patterns of phage genes in *B. subtilis*. Upon infection, phages go through a predictable pattern of early, middle (4–5 min) and late (8–10 min) gene expression (Gage and Geiduschek 1971). Phage genes transcribed in this temporal pattern are located on different strands (heavy or light) of the phage genome. Whilst these specific temporal and strand-specific transcriptional patterns are governed by phage-encoded proteins (Gage and Geiduschek 1971; Fox 1976; Giacomoni 1981), it has been shown that δ, as an innate part of RNAP, is also involved in maintaining their specificity and order of transcription (Pero, Nelson and Fox 1975; Achberger, Hilton and Whiteley 1982a; Dobinson and Spiegelman 1987). These experiments initially demonstrated that δ has a supportive role for transcriptional selectivity, acting alongside and in concert with other transcription factors (e.g. phage-encoded σ factors and regulatory proteins). This was projected to not only be true for phage-related genes but for the transcriptional process in general.

The δ subunit enhances RNAP fidelity and selectivity

Other early studies on the function of δ centered not only around phage genes but also on its effects on transcription from synthetic templates (poly(dA-dT)) (Tjian *et al.*, 1977). It was reported that whilst δ had only a limited effect on the expression of phage genes, it strongly decreased RNAP activity towards synthetic templates, suggesting a general function in selectivity. This led to studies focused on a wider spectrum of transcriptional targets, employing a variety of templates, including different phages, *B. subtilis* chromosomal DNA, plasmids and synthetic targets (Dickel, Burtis and Doi 1980). These *in vitro* transcription experiments revealed varying effects after the addition of purified δ to reaction mixtures. Specifically, in the case of one phage tested (Φe), δ had no negative effects on RNAP activity, whilst for *B. subtilis* chromosomal DNA, the synthesis of RNA was decreased by up to 87% for some regions in the presence of this subunit. Moreover, transcription from DNA fragments that did not contain specific and known promoter sequences was almost entirely repressed by the presence of δ. This suggests that the δ subunit affects different promoters in different ways, thus supporting promoter specificity of the transcriptional machinery, whilst at the same time suppressing transcription from loci that do not contain δ-specific promoter elements. Similar effects for δ on the transcription of non-coding-regions were also described by Achberger and Whiteley (1981). Their studies, using *in vitro* transcription employing endonucleolytic fragmented DNA from *B. subtilis* bacteriophage SP82, revealed that in the absence of δ, the RNAP holoenzyme binds non-specifically to fragments that do not have promoters, resulting in transcription from these sites. The addition of δ to *in vitro* transcription reactions restored binding of the complex exclusively to promoter regions and produced transcription patterns (mid vs late phage genes) that are characteristic of those seen *in vivo*. Along the same lines, UV cross-linking experiments reveal that the addition of δ to core-RNAP or the RNAP holoenzyme leads to decreased binding at non-promoter sequences (Hilton and Whiteley 1985).

In a study by Dobinson and Spiegelman (1987), features that guide the variable effects of different promoters were explored by assessing the expression of two early Φ29 promoters in run-off experiments (initiation complex assays). In these works, it was shown that the influence of δ on transcription appears to be dependent on the strength of the promoter in question. Specifically, varying effects on transcriptional repression were observed, with the stronger of the two promoters showing no decrease in expression upon δ addition, whilst the weaker promoter resulted in a 50% decrease in transcription after supplementation with the subunit. These results gave early insight into the selective effects of δ, and suggested that promoter strength may be a driving force of its activity. This model has been supported by the findings of Juang and Helmann (1994b) in their work with the *B. subtilis ilv-leu* operon. In this study, it was shown that δ negatively influences open complex formation and has stronger effects towards the transcription of weaker promoters, and less of an influence on medium strength promoters. Consequently, it was suggested that δ decreases promoter melting, which leads to decreased transcription from non-promoter sequences, as well as at weaker promoters. In contrast, promoters that are considered closer to optimal are relatively independent of the effects of δ. Thus, a model describing three general types of promoters and their susceptibility to δ was proposed as follows (Juang and Helmann 1994b): (i) at weak promoters, δ prevents RNAP binding and therefore transcription; (ii) at medium strength promoters, δ allows the binding of RNAP, but decreases open complex formation; and (iii) at strong promoters, δ seemingly has little effect on gene expression.

δ interferes with open complex formation

Initial Studies by Spiegelman, Hiatt and Whiteley (1978) and Achberger, Hilton and Whiteley (1982a) indicated that δ functions in the window between promoter recognition and initiation, rather than being directly involved in initiation itself. Subsequently, experiments by Chen and Helmann (1997) furthered this notion, showing that δ is important for open complex

formation. This is in support of Juang and Helmann, who used, amongst other approaches, footprinting analysis to describe the inhibitory effects of δ on open complex formation (Juang and Helmann 1994a, 1994b, 1995). Furthermore, they demonstrated that loss of δ specifically interfered with promoter melting, rather than impacting the stability of the open promoter complex, which is somewhat in contrast to Rabatinova *et al.* (2013), who suggested that the longevity of open complexes is diminished by δ. Importantly, each of these studies again support the notion that δ influences transcription prior to initiation.

RNAP recycling

Whilst the majority of δ-related studies describe its negative effects on transcription of specific promoters, several groups have demonstrated that δ also has the capacity to increase overall transcriptional activity (Spiegelman, Hiatt and Whiteley 1978; Achberger and Whiteley 1981; Juang and Helmann 1994b). These positive effects are suggested to be a result of a decrease in non-specific transcription, as well as increased core-RNAP recycling in the presence of δ. The specific mechanism by which this latter process occurs is not fully understood; however, a more efficient release of RNAP after termination of transcription has been suggested (Juang and Helmann 1994b). This effect is thought to parallel the manner by which δ prevents RNAP from binding to non-promoter sequences, thereby leading to decreased product inhibition, and increasing the speed at which the transcription complex is available to initiate new rounds of gene expression. Structurally, it has been hypothesized that this process is facilitated by the C-terminus of δ (Lopez de Saro, Woody and Helmann 1995), where the negatively charged part of the protein competes with DNA/RNA for RNAP binding, thereby causing increased release of the transcriptional machinery. In more recent experiments investigating the function of HelD, another RNAP-associated protein, it was confirmed that δ is able to increase transcriptional cycling by faster release of RNAP after termination; and is also able to release stalled RNAP from DNA, increasing transcriptional activity (Wiedermannova *et al.*, 2014). Both effects have also been found to be true for HelD, and interestingly, both proteins together show a strong synergistic effect. Whilst the precise reason for this synergy is incompletely understood, the results highlight the complex and multifactorial regulation of RNAP activity.

The role of the initiating nucleotide on δ-dependent regulation

Although the contention that δ-dependent transcriptional effects are influenced by individual promoter strength is well established, it fails to completely explain all changes in gene expression mediated by this subunit. Therefore, a more recent study in *B. subtilis* has centered on delineating the exact role of δ in promoter melting and open complex formation, with a specific focus on the transcription initiating NTP (iNTP) (Rabatinova *et al.*, 2013). This idea was shaped by previous works, showing that promoter activity is not only determined by pure binding efficiency of RNAP to promoter sequences but also by the ability to initiate transcription. While high levels of iNTPs ensure efficient transcription, lower amounts cause collapse of the open promoter complex, and prevent transcriptional initiation. In *Escherichia coli*, transcriptional initiation from rRNA promoters, which have inherently unstable open promoter complexes, is dependent on the availability of transcript specific iNTPs (ATP or GTP) (Gaal *et al.*, 1997; Murray, Schneider and Gourse 2003). A similar scenario was also shown for *B. subtilis*, where nutritional starvation and onset of the stringent response results in decreased levels of GTP and therefore negatively influences transcription of iNTP-sensitive rRNA promoters (Ochi, Kandala and Freese 1982; Krasny and Gourse 2004). Subsequently, it was shown that such regulation is not only true of rRNA promoters but other genes as well, thus presenting a general regulatory concept (Krasny *et al.*, 2008). Accordingly, Rabatinova *et al.* (2013) explored the influence of iNTP on δ-mediated function in *B. subtilis*, revealing that the δ subunit influences transcription by enhancing the effects of iNTP on open complex formation. Promoters that possess relatively unstable open complexes require higher amounts of iNTP to initiate transcription. δ destabilizes open complex formation, thus increasing the amount of iNTP required for successful transcription initiation; supporting the notion of iNTP-dependent transcriptional regulation. This appears to be particularly true for promoters that are iNTP sensitive (e.g. rRNA promoters), whilst other promoters, which display stable open complexes, are less likely to be affected by the influence of the δ subunit.

The impact of abrogated δ activity on fitness and virulence

Phenotypic effects of δ deletion

Although the specific effects of δ on transcription have been well documented, uncovering the phenotypic effects of *rpoE* deletion was, at least initially, more challenging. A *B. subtilis* mutant lacking the δ subunit of RNAP was shown to be viable, able to form spores and did not display obvious growth defects under standard laboratory conditions (Lampe *et al.*, 1988). Although unexpected at first, these results are not surprising for an accessory subunit. They do, however, raise the question about under which conditions the transcriptional effects of δ result in altered behavior of the cell, and, thus, under which stimuli the δ subunit is beneficial for cellular survival. An early indication about possible δ function came from a study in *S. aureus* investigating starvation survival. Watson, Antonio and Foster (1998) identified *rpoE* during a transposon screen targeted towards genes that are important for survival and recovery from prolonged stationary phase starvation. A transposon insertion within the early portion of *rpoE* caused impaired survival under amino acid-limiting conditions and acid stress. Similar *rpoE*-dependent stress phenotypes were later shown in *S. mutans*, where a δ subunit mutant is impaired in acid and H_2O_2 stress survival (Xue *et al.*, 2010). Additionally, the mutant was described as having a clumping phenotype, and to reach lower final densities in liquid cultures when compared to the wild type. Another common phenotype described for various bacterial species, including *B. subtilis* (Lopez de Saro, Yoshikawa and Helmann 1999), *S. aureus* (Weiss *et al.*, 2014) and *S. agalactiae* (Jones, Needham and Rubens 2003), is the extended lag phase of *rpoE*-depleted strains when subcultured into fresh media from stationary phase growth. Thus, it would appear that δ is required for adaption to, and survival during, stress and changing environmental conditions, as well as growth phase transition. It has also been documented that *B. subtilis rpoE* mutants are characterized by elongated cell morphology, the propensity to clump during log phase and the appearance of rough-edged colonies when grown on solid media (Lopez de Saro, Yoshikawa and Helmann 1999). Interestingly, and despite earlier studies that described normal sporulation of *B. subtilis* Δ*rpoE* strains (Lampe *et al.*, 1988; Lopez de Saro, Yoshikawa and Helmann 1999), it has also been shown that a transposon insertion in this gene suppresses the effects caused by mutations (*pdhB* or *pdhC*) that block sporulation (Gao and Aronson 2004). The exact mechanism by which this occurs is unclear; however, these findings emphasize the widespread effects

of δ on transcription, and its importance in maintaining cellular homeostasis. Considering the widespread defects displayed by *rpoE* mutants in a wealth of species, it is perhaps expected that *rpoE* mutants of *B. subtilis* are readily outcompeted by the wild-type strain when cultured together over prolonged periods (Rabatinova *et al.*, 2013).

The role of δ in pathogenic organisms

Several groups have examined the effects of *rpoE* deletion on more complex processes, such as during the interaction of pathogenic bacteria with their hosts. Since disease causation is a process that involves the precise timing of expression for a variety of different genes, including those required for interaction with the immune system, virulence factor expression, biofilm formation and nutrient acquisition, it is perhaps no surprise that δ has been shown to play and important role in virulence. In *S. agalactiae*, a study employing signature-tagged transposon mutagenesis in a neonatal rat sepsis model identified an *rpoE* mutant as displaying the most impaired virulence from the entire library (Jones, Knoll and Rubens 2000). In a subsequent study by the same group, it was suggested that the virulence defect was linked to increased killing by phagocytes, explaining the lower survival rates observed for *rpoE* mutants in whole human blood; despite not showing altered survival in human plasma and chemically defined media (Jones, Needham and Rubens 2003). An important consideration with these findings is that the *rpoE* transposon mutant is not a complete knockout, but is in fact only a low *rpoE* expressing strain due to the location of the insertion. As such, the strain possesses around a 10-fold decrease in *rpoE* transcript compared to the wild type, which is seemingly insufficient to maintain full δ-function, leading to the decrease in virulence described. Therefore, it appears that in *S. agalactiae*, and perhaps other pathogens (see below), there is a minimal concentration of δ required in the cell to ensure transcriptional specificity and facilitate virulence.

The pathogenic role of δ has also been explored in another streptococcus, namely *S. mutans*. Interestingly, and in contrast to the negative effects of *rpoE* loss on virulence that were obtained for *S. agalactiae*, it was described that deletion of *rpoE* leads to increased expression of virulence-related traits (Xue *et al.*, 2011). Specifically, increased co- and self-aggregation, and an altered extracellular matrix, have been reported for *rpoE* mutants, which may be caused by differentially expressed surface proteins and polysaccharides. Furthermore, *S. mutans* Δ*rpoE* strains also display elevated attachment to human extracellular matrix components, such as collagen and fibronectin. Despite these seemingly enhanced aggregative properties, *rpoE* deletion causes an inability to attach to human epithelial cells (HEp-2) compared to the wild-type strain; likely due to increased clumping of the mutant. Beyond these findings, loss of *rpoE* in *S. mutans* results in the derepression of enzymes that facilitate the metabolism of a number of sugars, allowing the mutant to utilize a larger array of carbon sources than the parental strain. Additionally, a panel of antibiotics and compounds that are toxic to the wild type were ineffective against the mutant in phenotypic microarray studies. This highlights the remarkably broad impact that δ has on bacterial physiology and metabolism, each of which influence cellular fitness, and thus pathogenesis.

In a more recent study by our group, we explored the effects of δ in the major human pathogen, *S. aureus* (Weiss *et al.*, 2014). Deletion of *rpoE* resulted in decreased expression and abundance of a variety of virulence factors, leading to decreased fitness of the strain and impaired virulence. This latter point is manifested by a diminished ability to survive in whole human blood, and resist phagocytosis by human leukocytes. These *in vitro*/*ex vivo* phenotypes were shown to correlate with *in vivo* findings, where the mutant strain was found to be significantly impaired in its ability to cause disease in a murine model of sepsis and dissemination.

Global transcriptional and translational effects of δ

Exploring the influence of δ on global gene expression has been aided by cutting edge high-throughput methodologies, including transcriptomics and proteomics. These techniques have provided insight into the role of δ in maintaining transcriptional specificity, and how this links to the various phenotypes observed. To explore why an *rpoE* mutant of *S. mutans* displays alterations in virulence-related behaviors, as well as impaired ability to survive during stress (Xue *et al.*, 2010, 2011), gene expression (Xue *et al.*, 2010) and protein abundance (Xue *et al.*, 2012) were assessed for the wild type and mutant under several conditions. Transcriptomic analyses conducted via microarray revealed that, independent of the growth condition, more than 50% of the upregulated transcripts in an *rpoE* mutant were non-coding regions (Xue *et al.*, 2010). In comparison, only 20–30% of non-coding transcripts displayed increased expression in the mutant strain. These findings corroborate previous *in vitro* studies that demonstrate non-specific transcription of non-promoter regions when δ is removed from core RNAP (see above).

In addition to its effects on non-coding regions, coding sequences were also shown to be influenced by δ in *S. mutans*, in a manner partially independent of growth phase or condition (Xue *et al.*, 2010). Specifically, 24 genes were identified as being downregulated in the *rpoE* mutant under all conditions. These genes were involved in malolactic fermentation, histidine metabolism, biofilm formation, adherence, virulence, and resistance to antibiotics and other inhibitory compounds. In addition, during growth under standard laboratory conditions, a large number of transcripts were differentially expressed when comparing the mutant and parental strain, including those involved in the transport of a wealth of different compounds, metabolism and energy production/conversion. Following H_2O_2 or acid stress, a number of protective genes were not induced in the mutant strain, indicating that an incomplete stress response exists upon *rpoE* loss; however, the general stress response still appeared to be intact in the mutant strain. The authors ultimately concluded from these works that diminished survival of the mutant strain when exposed to stress results from a general deregulation of the *S. mutans* transcriptome, leading to an impaired, slower and less directed attempt to adapt to changing environments and stress conditions.

In follow-up studies, the same group compared previous transcriptomic data with new proteomic data of the *rpoE* mutant and wild type, using identical growth conditions (Xue *et al.*, 2012). It was noted that two proteins, phosphoglucomutase (PGM) and phosphopentomutase (DeoB), were decreased in abundance either during all (PGM) or most (DeoB) growth phases and conditions. Both were identified as displaying this same pattern in microarray analysis as well, suggesting distinct and very gene-specific regulatory effects of δ. In the context of growth phase, chaperones, stress-related factors and enzymes known to be involved in protein turnover were altered in the mutant during exponential phase; whilst multiple sugar transporters and metabolism (MSM) systems demonstrated fluctuations during stationary phase. It was suggested that these changes in protein abundance could be an indicator of internal cell stress, rather than direct *rpoE*-guided effects. When comparing protein

composition of the *rpoE* mutant and parental strain after exposure to external stressors, it was noted that both strains were able to induce protection-specific pathways, which was again largely consistent with transcriptomic data. However, despite general adaptation patterns being similar between mutant and wild type, it was observed that the upregulation of proteins required to adapt to environmental change was weakened upon the loss of δ, again indicating a slower or dampened response to stress.

Using RNAseq technologies, our own group revealed similar effects of *rpoE* deletion in *S. aureus* as compared to *S. mutans* (Weiss *et al.*, 2014). Specifically, our *rpoE* mutant displayed differential expression of 191 genes compared to the wild type during exponential growth, including a significant decrease in many known or implicated virulence factors, such as toxins, hemolysins and secreted proteases. While global regulators were largely unaffected, the clustered alteration of virulence gene expression again suggests gene specific effects, such as those seen for PGM and DeoB in *S. mutans* (Xue *et al.*, 2012). Beyond reduced virulence gene expression, we notably observed that under standard conditions weakly expressed genes were generally derepressed upon loss of *rpoE*. Interestingly, we also saw a large increase in non-core genome elements, including prophage-encoded genes. This parallels the effects seen for non-coding sequences in *S. mutans*, and further highlights that loss of the δ subunit results in decreased transcriptional selectivity, and an overall deregulation of the transcriptional process.

When one considers all of the available transcriptomic/proteomic studies with *rpoE* mutants, three general conclusions can be made. (i) Certain genes and gene groups are changed in expression/protein abundance independent of growth phase or stress condition. This suggests a gene-specific mechanism of regulation that involves δ, opening the possibility that there may be promoter features other than strength (for example iNTP-sensitivity) at work. (ii) During exponential and stationary phase growth (as seen for *S. aureus* and *S. mutans*), loss of *rpoE* leads to a complex deregulation of transcription, causing differential expression patterns, and the upregulation of typically lowly expressed genes and non-coding elements. (iii) Finally, it has been shown that cells lacking *rpoE* still adapt to changing environmental conditions, but in a less rapid and less robust manner than the wild type. This would seem to account for the decreased survival of *rpoE* mutants under stress conditions, and also explain the extended lag phase observed when mutants are subcultured into fresh media. Accordingly, the effects of *rpoE* disruption on survival and adaptation can be viewed as a fitness defect, resulting from diminished transcription of specific pathways and traits due to relaxed transcriptional specificity. With this being said, the specific and direct regulation of certain genes (e.g. PGM and DeoB in *S. mutans*) and traits (virulence determinants in *S. aureus*) still occurs. Collectively, each of these facts and findings highlight the multifactorial influence of δ, ranging from gene-specific to global effects, leading to a lack of fitness, and alterations in key cellular processes.

Summary of the δ subunit

Collectively, all of the information contained herein leads to a model for δ function (Fig. 2): A major feature of the δ subunit is that it decreases the stability of RNAP-DNA interaction at non-coding regions and weak promoters that possess inherently unstable open promoter complexes. Consequently, δ biases the transcriptional machinery towards those open-reading frames that have strong promoters/stable open promoter complexes, thus favoring expression from such sites. In addition to this, features beyond promoter strength (e.g. iNTP requirements) likely result in promoter-specific effects of δ in addition to its more general role. Furthermore, δ increases RNAP recycling and causes overall enhanced transcriptional activity. In

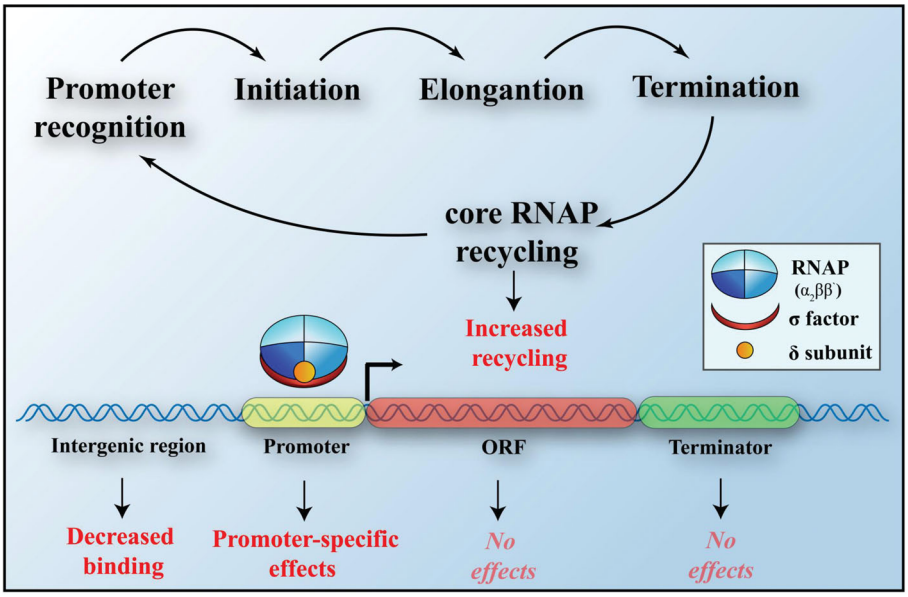

Figure 2. A model describing the effect of δ on different phases of transcription. The available literature suggests that δ has varying effects on each stage of the transcriptional process. The subunit prevents non-specific binding and transcription of RNAP from intergenic and non-promoter-containing regions. This influence on open complex formation and the initiation of transcription vary from promoter to promoter, thereby increasing transcriptional specificity of RNAP. Whilst elongation and termination are seemingly unaffected, δ does limit the binding of RNAP to DNA at terminators, allowing faster recycling of the enzyme, and increasing overall transcription. Shown are the stages of transcription and the corresponding gene regions in black letters and δ-dependent effects in red. Note that the ε and ω subunits are omitted from this figure for reasons of simplicity.

general, δ weakens the binding of RNAP to DNA and negatively influence open complex formation and stability; which appears to be mediated by structural features of the δ protein. Specifically, its N-terminus mediates interaction with core RNAP, whilst the negatively charged and intrinsically disordered C-terminus is thought to interfere with the RNAP-DNA interaction. Ultimately, δ acts as a guide for RNAP, increasing its transcriptional specificity. Loss of this factor causes deregulation, and results in a decreased ability to adjust to changing environments, general fitness defects and the reduced virulence of pathogenic species. All of these consequences highlight the central importance of δ as a regulatory factor directing gene expression in Gram-positive bacteria.

ADDITIONAL, SMALL ACCESSORY RNAP SUBUNITS IN GRAM-POSITIVE BACTERIA

In addition to the δ subunit, Gram-positive bacteria possess two other accessory RNAP subunits, omega, ω (RpoZ) and epsilon, ε (RpoY). Whilst ε is unique to Gram-positives, ω is found in RNAP complexes from archea to eukaryotes. Both of these subunits and their roles within the RNAP complex is discussed in the following sections.

The ω subunit

The ω subunit of RNAP is an intensively studied protein that has been widely described in the literature. With this being said, the majority of studies have been performed using the Gram-negative model organism *E. coli*, and appear to be only partially translatable to Gram-positive species, as discussed below. An extensive and comprehensive review on the history of ω research, including Gram-positive as well as Gram-negative bacteria, has been published by Mathew and Chatterji (2006) where the authors summarize structural and functional roles of the subunit. In the context of this review, we will offer an overview of data that is available and valid for Gram-positive organisms, as well as highlight functional differences compared to Gram-negative bacteria.

The ω protein is widely conserved across all forms of life

With an increasing number of bacterial, archaeal and eukaryotic genomes available, it has become clear that the ω subunit is conserved in all branches of life. In eukaryotes, a sequence, structural and functional homolog of ω (RPB6) exists that is associated with RNAPI, II and III, whilst archaeal genomes harbor a sequence homolog termed RpoK (Minakhin *et al.*, 2001). RPB6 has been shown to be essential for RNAP assembly in eukaryotes (Nouraini, Archambault and Friesen 1996); however, in bacteria, deletion of the ω subunit in a variety of different species does not result in cell lethality (Gentry and Burgess 1989; Kojima *et al.*, 2002; Mathew, Ramakanth and Chatterji 2005; Doherty *et al.*, 2010; Jie, JiLiang and DongJie 2010; Santos-Beneit *et al.*, 2011; Gunnelius *et al.*, 2014). This is particularly intriguing, since the subunit is conserved in all of the sequenced genomes of free-living bacteria (Minakhin *et al.*, 2001), suggesting an important evolutionary role for it in cellular survival

The binding of ω to RNAP

The ω protein, which ranges in size from ∼9 to 11.5 kDa depending on the bacterial species in question, was first shown to be a dedicated subunit of *E. coli* RNAP, rather than just a tightly bound factor, when cross-linking experiments demonstrated its specific binding to the β′ subunit (Gentry and Burgess 1993). Subsequently, it was found to be an integral part of the transcription machinery *in vivo* when the association of ω directly with RNAP was shown (Dove and Hochschild 1998). In these studies, ω was translationally fused to a DNA-binding protein (the cI repressor from bacteriophage λ) and was found to activate transcription of promoters adjacent to the corresponding λ operator, demonstrating interaction of the chimeric λcI-ω fusion protein with RNAP. Both of these studies have since been supported by more recent work solving the structure of ω-containing RNAP from *Thermus aquaticus* and *E. coli* (Zhang *et al.*, 1999; Murakami 2013). Although no such crystallization studies for RNAP have been performed in Gram-positive bacteria, several experiments in *B. subtilis* demonstrate co-purification of ω with RNAP, highlighting its association with other subunits of the transcriptional machinery (Spiegelman, Hiatt and Whiteley 1978; Achberger, Tahara and Whiteley 1982b). These results were confirmed and extended in a recent approach in which Delumeau *et al.* (2011) were not only able to co-purify ω with the essential RNAP subunits (α, β, β′) but also determined a 2:1:1:1 ratio for $\alpha_2\beta\beta'\omega$ during exponential and stationary growth. These results clearly show that, as for Gram-negative bacteria, the ω subunit is an integral part of the Gram-positive transcriptional machinery.

The role of ω for structural integrity of RNAP

Extensive research has been performed on the involvement of ω in folding of the β′ subunit, and RNAP assembly. Its importance for the assembly process was first considered when RNAP purified from *Rhodobacter capsulatus* and *E. coli* was shown to require ω, or GroEL and ω, respectively, to remain active *in vitro* (Mukherjee and Chatterji 1997; Richard *et al.*, 2003). This requirement for the GroEL chaperone was considered puzzling, but this factor had been shown to co-purify with RNAP, and was thus thought to be important for the folding of the transcriptional complex (Mukherjee and Chatterji 1997; Mukherjee *et al.*, 1999). Interestingly, deletion of ω in *E. coli* results in a massive increase of GroEL recruitment to RNAP, suggesting that GroEL might be employed to overcome misfolding due to ω loss (Mukherjee *et al.*, 1999). This was confirmed when *in vivo* GroEL substrates in *E. coli* were determined (Houry *et al.*, 1999), showing that other than β′, all of the essential RNAP subunits, as well as ω, require GroEL for proper folding. Surprisingly, these results were found to be only partially true in *B. subtilis*, where GroEL does not appear to be associated with RNAP subunits (Endo and Kurusu 2007). With this being said, the Gram-positive study identified considerably fewer GroEL substrates than the study performed in *E. coli* (28 vs ∼300). Therefore, either there are indeed fewer substrates for this chaperone in *B. subtilis* or experimentation in the Gram-positive organism had lower sensitivity in comparison with the *E. coli* study.

Interestingly, an earlier study in *E. coli* described *in vitro* cross-linking of ω and β′ (Gentry and Burgess 1993), whilst at the same time showing that β′ was not a substrate of GroEL and that the ω subunit increases (together with GroEL) the *in vitro* activity of RNAP. As such, it was suggested that ω had a specific function in supporting the folding of β′. This hypothesis was reinforced by a structural study that investigated the precise interplay of ω and β′, demonstrating interaction of conserved regions for these two proteins (Minakhin *et al.*, 2001). Consequently, it was proposed that ω acts as a molecular latch, guiding the interaction of β′ with the $\alpha_2\beta$ complex. Studies by Ghosh, Ishihama and Chatterji (2001) supported this notion by demonstrating that the interaction of ω with β′ was able to prevent aggregation of the latter protein during *in vitro* renaturation. Furthermore, this same group was able to show that native folding of β′ requires ω, and

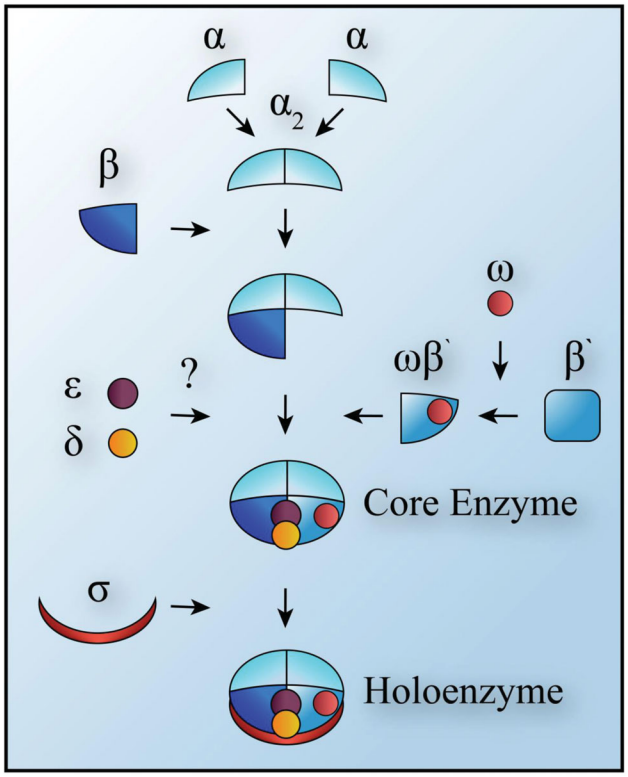

Figure 3. Model for RNA polymerase assembly in Gram-positive bacteria. After association of two α subunits, the dimer binds to the β subunit. The β' protein is held by the ω subunit (Minakhin *et al.*, 2001) in order to prevent misfolding (Ghosh, Ramakrishnan and Chatterji 2003) and aggregation (Ghosh, Ishihama and Chatterji 2001), before the β'-ω complex docks with $\alpha_2\beta$ (Mathew and Chatterji 2006; Ganguly and Chatterji 2011). Thus far, it is unknown when the ε and δ subunits bind to the complex; however, it is believed that ε interacts at the region of the β' jaw and secondary channel (Keller *et al.*, 2014), whilst δ is thought to interact with the β and β' subunits (Weiss *et al.*, 2014). Finally, and in order to enable RNAP to perform promoter-specific transcription, the σ factor docks with core RNAP resulting in holoenzyme formation.

that folding of the β' subunit in the absence of ω results in an intermediate state between a denatured and native conformation (Ghosh, Ramakrishnan and Chatterji 2003). These results were supported by work in *Bacillus cereus*, where expression of ω increased by as much as 10-fold after heat stress (42°C), suggesting a chaperone-like role, where larger amounts of the subunit can counteract RNAP denaturation (Periago *et al.*, 2002). Other than a function in β'-folding, it was also shown that the removal of ω leads to increased proteolytic cleavage of β' during RNAP isolation in *Mycobacterium smegmatis*, suggesting an additional protective role of the ω subunit (Mathew, Ramakanth and Chatterji 2005). Based on these studies, the involvement of ω in RNAP assembly was described, whereby an α-dimer and the β subunit assemble before docking to ω-bound β' occurs (as visualized in Mathew and Chatterji 2006 and shown in Fig. 3). Both the sequential assembly of the subunits themselves, as well as the importance of ω for the assembly process, was recently confirmed by studies that investigated active RNAP reconstitution at air–water interfaces (Ganguly and Chatterji 2011).

Further to this, the comparison of different RNAP crystal structures revealed that ω seems to have varying interactions with β' and within the RNAP complex in diverse organisms (Murakami 2013). For example, in *T. aquaticus* and *E. coli*, ω displays only partially overlapping conformations, and thus has varying interactions with β'. This is especially interesting, given that no complete Gram-positive RNAP structure is available so far, and therefore no definite assumptions about $\omega - \beta'$ interaction can be made. These differences in conformation and interaction between the subunits could account for the various functional properties of ω in different bacterial species, which are discussed in the following section.

ω and the stringent response: structural and functional variability in different bacterial species

In *E. coli*, the ω-encoding *rpoZ* gene is located in the same operon as *spoT* (Gentry and Burgess 1989; Sarubbi *et al.*, 1989). SpoT is a bifunctional enzyme that facilitates the degradation and synthesis of guanosine tetraphosphate and pentaphosphate (ppGpp and pppGpp), thus regulating the levels of these alarmones, and onset of the stringent response (reviewed in Cashel *et al.*, 1996; Chatterji and Ojha 2001). In such a scenario, ppGpp/pppGpp are produced as a result of nutritional stress and cause global changes in transcription. Extensive work has centered on the mechanism of ppGpp/pppGpp recognition by ω, largely in *E. coli* (reviewed in Mathew and Chatterji 2006). Such studies recently led to identification of the 'magic spot', the binding site of ppGpp on RNAP (Kahrstrom 2013). Together three publications by different groups described that the binding of ppGpp to RNAP occurs at the interface of the β' and ω subunits (Mechold *et al.*, 2013; Ross *et al.*, 2013; Zuo, Wang and Steitz 2013), and that deletion of key residues negatively influences growth under nutrient-limiting conditions via loss of ppGpp binding to RNAP (Ross *et al.*, 2013). Interestingly, homology analysis shows that key β' and ω residues important for ppGpp binding are not conserved within the Firmicutes (or the *Thermus* and *Aquificales* genera), therefore suggesting alternative mechanisms of ppGpp recognition by RNAP exist (Ross *et al.*, 2013). This is in line with findings from *B. subtilis*, highlighting that although ppGpp is used as a signaling molecule to react to changing nutrient availability, ppGpp does not change transcriptional specificity by directly binding to ω and RNAP; thus, ω deletion does not interfere with stringent response induction (Krasny and Gourse 2004; Doherty *et al.*, 2010; Kriel *et al.*, 2012). Instead, this alarmone causes changes in intracellular GTP concentrations by modulating the activity of GTP synthesis proteins, which subsequently leads to altered activity of GTP (iNTP) sensitive promoters, such as ribosomal RNA genes (Krasny and Gourse 2004; Kriel *et al.*, 2012). Together these findings show that, although ω and the utilization of ppGpp to signal and react to nutrient limitation are conserved, the mechanism for alarmone recognition is variable. This emphasizes the need to examine and understand the function of ω in species beyond the standard model organism, *E. coli*. Interestingly, these possible functional differences between genera are also mirrored in the cellular abundance of the subunit itself. Doherty *et al.* (2010) were able to show that in *E. coli* almost every RNAP polymerase includes a ω subunit (85%), whilst in *B. subtilis* the amount is significantly lower, with only 48% of RNAP complexes containing the subunit.

ω is involved in σ factor recruitment

RNAP purified from *E. coli rpoZ* mutants displays an altered composition, with the amount of σ^{70} associated diminished, and a concomitant increase in bound σ^{38} (Geertz *et al.*, 2011). These changes in RNAP composition result in altered gene expression and DNA relaxation. Interestingly, the wild-type phenotype (increased supercoiling) is restored by overexpressing σ^{70} in ω-null strains. Similar results were obtained in the cyanobacterium *Synechocystis* sp. PCC 6803 (Gunnelius *et al.*, 2014a), wherein an

rpoZ knockout had impaired recruitment of the primary σ factor, resulting in decreased expression of genes under the control of this element. Inversely, a large number of typically lowly expressed genes were upregulated, suggesting activity of one or more alternative σ factors. These data indicate a role for ω in transcriptional specificity, and are supported by later publications describing an altered response to heat stress upon deletion of *rpoZ*, leading to impaired survival at higher temperatures (Gunnelius *et al.*, 2014b). Importantly, such findings correlate with early studies showing that purification of ω-deficient RNAP in *E. coli* is depleted for σ^{70}, leading to the conclusion that ω is required for correct RNAP folding and σ-factor binding (Mukherjee *et al.*, 1999). Overall, this line of research describes how the structural effects of ω are required for RNAP integrity and functionality, which leads to a role in fine-tuning transcription by adjusting affinity for central transcriptional regulators such as σ factors. These results were all obtained in Gram-negative organisms, thus highlighting the need for further investigation in Gram-positive species.

The ω subunit of Gram-positive bacteria

As detailed above, in *E. coli* the ω subunit mediates folding of the β' subunit, influences assembly of RNAP, orchestrates gene expression during the stringent response and plays a role in ordering transcriptional specificity. In Gram-positive bacteria, the functional role of ω is much less clear. Since ppGpp does not bind RNAP in *B. subtilis*, the ω subunit is unlikely to be directly involved in the stringent response of this organism. This raises the question of additional functions for the Gram-positive ω subunit outside of ppGpp binding and β' folding. Whilst studies have been conducted for various Gram-negative bacteria beyond *E. coli*, including *Sinorhizoboium meliloti* (Krol and Becker 2011), *Xanthomonas campestris* (Jie, JiLiang and DongJie 2010) and cyanobacteria species (Gunnelius *et al.*, 2014a, 2014b), thus far the role of ω in Gram-positive bacteria has only been extensively examined in two *Streptomyces* species and *M. smegmatis*.

When exploring the role of the ω subunit in *Streptomyces kasugaensis*, it was found that an *rpoZ* mutant had diminished synthesis of the antibiotic kasugamycin, and that the strain was also deficient for aerial hyphae generation (Kojima *et al.*, 2002). A genetic basis for this latter phenotype was not reported; however, the kasugamycin biosynthetic genes were found to have decreased expression in the mutant strain. This decrease in expression appears to be mediated by reduced production of the KasT regulator, which is known to positively regulate kasugamycin production (Ikeno *et al.*, 2002, 2006). Interestingly, when *kasT* was expressed under the control of a different promoter (*ermEp*), it was transcribed at wild-type levels in the *rpoZ* mutant, and resulted in expression of the kasugamycin-producing operon (Kojima *et al.*, 2002, 2006). Although the underlying mechanism for this regulation is not currently understood, these results do indicate that ω is capable of having promoter-specific transcriptional effects.

Subsequent to this, the role of ω was also analyzed in *Streptomyces coelicolor*. Interestingly, the ω proteins of *S. kasugaensis* and *S. coelicolor* display 98% sequence similarity, with the protein from the latter being able to complement *rpoZ* loss in *S. kasugaensis* (Kojima *et al.*, 2002). Santos-Beneit *et al.* (2011) have detailed pleiotropic phenotypes for a *S. coelicolor rpoZ* mutant, including slow growth, absent spore-pigmentation and increased sensitivity to heat, with temperatures above 40°C leading to an aerial hyphae-null phenotype. Additionally, the mutant displayed altered expression of two antibiotics, actinorhodin (ACT) and undecylprodigiosin (RED). Whilst ACT was produced earlier during growth in an *rpoZ*-depleted strain, the mutant failed to produce the antibiotic in later growth phases, showing overall reduced levels after 70 h. For RED, it was reported that production was essentially abolished in the mutant strain, akin to kasugamycin synthesis in *S. kasugaensis*. While there is no clear explanation for how deletion of *rpoZ* simulatiously modulates expression of these structurally unrelated metabolites, or leads to pleiotropic phenotypic alterations in *S. coelicolor*, it was shown that *rpoZ* has a PhoP-binding site (PHO box) (Sola-Landa *et al.*, 2005) upstream of its transcriptional starts site (Santos-Beneit *et al.*, 2011). The repressor PhoP is known to inversely regulate the expression of secondary metabolites in response to the availability of phosphate (Sola-Landa, Moura and Martin 2003). For *rpoZ*, it was shown that PhoP does indeed bind to its promoter region, and that deletion of *phoP* results in an increased abundance of the subunit. Whilst these experiments suggest that ω itself can be regulated in an environment-depenent manner, it is not completely understood how the subunit exerts its effects on a plethora of different genes and cellular processes. Furthermore, the notion that *rpoZ* expression is controlled by an environment-responsive regulator is challenged, at least in part, by findings in *B. subtilis*. In this organism, Delumeau *et al.* (2011) described that during sporulation, or following stress in vegetative cells, no changes in RNAP subunit stoichiometry are observed. This is in line with findings from Nicolas *et al.* (2012), who report that expression of the subunit is stable under 104 different growth conditions in *B. subtilis*, suggesting constant and environment-independent abundance of ω. These opposing findings may represent species-specific differences, highlighting not only the need to investigate how ω mediates its effects on gene expression but also how its production within the cell is controlled.

In *M. smegmatis*, it was first shown that *rpoZ* deletion causes changes in colony morphology, and a slower growth phenotype similar to that reported for *E. coli* (Mukherjee *et al.*, 1999; Mathew, Ramakanth and Chatterji 2005). Further studies described that ω-depleted strains present additional pleiotropic alterations, including a decrease in sliding motility, and diminished biofilm formation due to an altered biofilm matrix and cell surface resulting from shorter chain mycolic acids (Mathew *et al.*, 2006). With regard to this latter point, ω-deficient strains of *M. smegmatis* are unable to manipulate their mycolic acid and glycopeptidolipide profiles to that required for biofilm maturation; indeed, even after the onset of biofilm formation, *rpoZ* mutants maintain mycolates only found in the wild type during planktonic growth (Mathew *et al.*, 2006; Mukherjee and Chatterji 2008). Two possible explanations for these findings have been suggested: firstly, the onset and maturation of biofilms requires significant changes in transcription (Sauer 2003), which are facilitated by various regulatory mechanisms, including alternative σ factors (Rachid *et al.*, 2000; Bateman *et al.*, 2001; Knobloch *et al.*, 2001, 2004). Secondly, such events are governed by stress adaption through the stringent response and ppGpp (Balzer and McLean 2002; Taylor *et al.*, 2002; Lemos, Brown and Burne 2004; Gjermansen, Ragas and Tolker-Nielsen 2006; He *et al.*, 2012). Both of these processes are controlled by ω in Gram-negative species, although, as suggested above, there appears to be disparate function for this subunit between the two classes of bacteria. Indeed, an alignment of the ω protein between *M. smegmatis* and *E. coli* reveals that the conserved residues required for ppGpp binding are, as with *B. subtilis* (Ross *et al.*, 2013), not conserved (our unpublished observation). As such, it seems more probable that, as with *E. coli* and cyanobacteria, the binding of ω to RNAP in *M. smegmatis* results in an altered affinity for different σ factors, and therefore negatively influences the ability to express genes required for

biofilm formation. This highlights the variable nature and, more importantly, the need to study ω in a wider range of Gram-positive bacteria, before drawing general conclusions about its function.

The ε subunit

Besides the α_2, β, β', δ and ω, an additional seventh RNAP subunit was recently identified in Firmicutes. Early studies described a protein that co-purifies with RNAP in different *Bacillus* species (Spiegelman, Hiatt and Whiteley 1978; Achberger, Tahara and Whiteley 1982b), but not in *E. coli* (Achberger, Tahara and Whiteley 1982b). Since it had a similar size to the 9 kDA ω subunit (first termed ω_2), this additional 11 kDa protein was originally named ω_1. Subsequent studies in *B. subtilis* showed that the protein was expressed (Nicolas *et al.*, 2012) and associated with RNAP during different growth phases, and during environmental stress (Delumeau *et al.*, 2011). This protein was also shown to remain tightly bound when overexpressing the $\alpha_2\beta\beta'\omega$ RNAP complex (Yang and Lewis 2008), suggesting that it is indeed a real subunit, rather than just an RNAP-associated factor. This hypothesis was further supported by the finding that ω_1, as well as ω, is present in the cell at equal molecular amounts to β (Delumeau *et al.*, 2011).

Notably, since its initial description in the literature in 1978, no significant advances have been made to functionally and structurally characterize this protein, until recently. Acknowledging this lack of focus, Keller *et al.* (2014) investigated ω_1 in *B. subtilis* and confirmed its interaction with RNAP by examining co-localization of the protein with RNAP, proving that it actually functions as a true subunit. Furthermore, sequence analysis revealed that it is conserved only in the Firmicutes and shows no sequence similarity to the ω subunit. Therefore, the protein was recently renamed the epsilon subunit (ε), and its encoding gene termed *rpoY*. Interestingly, in Firmicute genomes sequenced to date, *rpoY* is located in a bicistronic operon with the gene encoding RNase J1 (*rnjA*). This RNase has been extensively described as being required for post-transcriptional regulation, including RNA degradation and maturation (Britton *et al.*, 2007; Deikus and Bechhofer 2011; Linder, Lemeille and Redder 2014). Although a connection for these two gene products has yet to be established, it is tempting to suggest that a functional importance may exist for both elements in posttranscriptional processes and/or transcript maturation.

When analyzing ε structure by X-ray crystallography, a $\beta\beta\alpha\beta$ motif that may putatively influence protein–protein interaction was described (Keller *et al.*, 2014). Interestingly, the structure of ε shows homology to Gp2 proteins, which are found in bacteriophages that infect Gram-negative bacteria (Hesselbach and Nakada 1977), and have been shown to block RNAP function by preventing open complex formation (Nechaev and Severinov 1999; Camara *et al.*, 2010; James *et al.*, 2012; Bae *et al.*, 2013). Indeed, similar to Gp2, it was shown that ε binds to the β' jaw and secondary channel of RNAP, potentially preventing access of phage proteins to the transcriptional machinery (Keller *et al.*, 2014). Although Gp2 is known to be present only in phages that infect Gram-negative bacteria, Keller and colleagues hypothesize that the ε subunit could represent a protective factor against thus far unidentified Gp2-like proteins of Gram-positive bacteriophages. This suggestion is in line with the increasing number of mechanisms that are being documented which protect bacteria against phage infection (Labrie, Samson and Moineau 2010). Even though the structural data derived by Keller *et al.* (2014) support such a hypothesis, no experimental evidence, including phage infection assays, has yet been established. Additionally, no phenotypes or effects on transcription *in vitro* or *in vivo* were reported upon ε deletion. This makes the ε subunit of RNAP an interesting target for future experimentation in *B. subtilis* and other Gram-positive bacteria in general.

CONCLUDING REMARKS

With the wealth of information available on small RNAP subunits in Gram-positive bacteria, it is clear that these proteins present an important part of the transcriptional machinery. Studies describing the role of δ, ε and ω have been conducted over several decades, and considerable progress has been made. These subunits have been shown by countless groups to possess vital functions, ranging from guiding the assembly and structural integrity of RNAP, σ factor selectivity, influencing promoter specificity and potential roles in protecting cells against phage infection. Their significance is mirrored by their presence and conservation within nearly all of the Gram-positive genomes thus far sequenced. With modern high-throughput transcriptomic and proteomic approaches, as well as structural studies, information has been brought to light that allows explanation of the numerous phenotypes observed for null mutants of the subunits in various bacterial species. Nevertheless, the underlying mechanism by which these proteins mediate their function is still incompletely understood. It will be fascinating to observe how new approaches and studies, combined with this existing body of knowledge, will guide future work to reveal the precise role of these subunits within bacterial cells.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSRE online.

ACKNOWLEDGEMENTS

We would like to thank William Broach, Christina Krute and Halie Miller for their review of the manuscript, as well as Galen Madden for help designing the illustrations.

FUNDING

This study was supported in part by grant AI080626 (LNS) from the National Institute of Allergies and Infectious Diseases.

Conflict of interest. None declared.

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